REMARKS

Applicants have amended claim 9 and added new claim 19. Upon entry of this amendment, claims 9 and 14-19 will be under examination.

Support for the amended claim 9 can be found in the specification, for example, Figure 1, page 14, lines 20-26, and page 19, Example 1. New claim 19 also is supported by the specification, see for example, page 1, lines 35-36. No new matter has been added.

The office action is discussed below.

Anticipation Rejection:

On pages 2-3 of the office action, the examiner has rejected the claims and alleged as being anticipated by both Ritterhaus *et al. (US Patent No. 6,193,979)* and Smith *et al. (US Patent No. 6,713,606)*. Applicants respectfully disagree with the examiner and traverse the rejection.

Applicants note that in order to reject a claim under 35 USC § 102, the examiner must demonstrate that each and every claim term is contained in a single prior art reference. *See Scripps Clinic & Research Foundation v. Genentech, Inc.*, 18 USPQ2d 1001, 1010 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 90 (Fed. Cir. 1986); *see also* MPEP § 2131 (Rev. 2, May 2004). Claim terms are to be given their plain meaning as understood by the person of ordinary skill in the art, particularly given the limitations of the English language. *See* MPEP §§ 707.07(g); 2111.01 (Rev. 2, May 2004). Claims are to be given their broadest reasonable interpretation consistent with applicants' specification. *See In re Zletz*, 13 USPQ2d 1320, 1322

(Fed Cir. 1989) (holding that claims must be interpreted as broadly as their terms reasonably allow); MPEP § 2111(Rev. 2, May 2004).

Not only must the claim terms, as reasonably interpreted, be present, an allegedly anticipatory reference must enable the person of ordinary skill to practice the invention as claimed. Otherwise, the invention cannot be said to have been already within the public's possession, which is required for anticipation. *See Akzo, N.V. v. U.S.I.T.C.*, 1 USPQ2d 1241, 1245 (Fed. Cir. 1986); *In re Brown*, 141 USPQ 245, 249 (CCPA 1964). Applicants review below the references with these concepts in mind.

The Ritterhaus *et al.* disclosure relates to a composition comprising complement proteins related to the complement receptor type 1 (CR1) and preferably in combination with the Lewis X antigen or the sialyl Lewis X antigen (see column 1, lines 16-25). The Smith *et al.* disclosure relates to soluble derivatives of soluble peptides that can be used according to the invention. The present claims, however, are not solely directed to such composition or derivatives, but rather inventive methods of use for soluble derivatives. Such claims are specifically permitted under 35 USC §§ 100(b), 101.

The cited references do not teach or suggest methods for preparing an organ by perfusion prior to transplantation or storage of the organ by providing an ischemic reperfusion injury prevention preparation for perfusion of an organ prior to transplantation or storage of the organ. Because the references do not concern such a method, thus, the references cannot anticipate the claims.

On page 2 of the office action, the examiner states that Ritterhaus *et al.* teach compositions that comprise complement-related protein (CR1) with Lewis X antigen or the sialyl Lewis X antigen, a carbohydrate moiety, thus anticipates

the claimed invention. In response, applicants provide the following in order to assist the examiner in distinguishing the claimed invention from the cited art:

Ritterhaus *et al.* refer to forms of soluble CR1 (sCR1), wherein the polypeptide chain contains modified glycoforms (including Le^x and sialyl Le^x) (see for example, col. 9, lines 58-66), which are not intrinsically membrane-interactive. The compositions of Ritterhaus *et al.* mediate binding to membranes only if a protein, for which these glycoform modifications are ligands, is expressed in a membrane-bound form on cells. The Ritterhaus *et al.* subject matter is based on such proteins (such as E-selectin), which can be up-regulated in certain tissues under certain conditions. However, Ritterhaus *et al.* provide no evidence that selectins are upregulated within a stored organ or that such upregulation is sufficient to bind enough sCR1 (sialyl Le^x) to protect the organ against complement attack. In fact, one skilled in the art would not have regarded this as probable that the disclosure of Ritterhaus *et al.* could provide such protection because Ritterhaus *et al.* would require the sCR1 derivative to be retained within the organ following flushing with excess preservation fluid and restoration of blood flow, (see for example, Takada *et al., J. Clin. Investigation*, 99, 2682-2690, 1997. Copy enclosed). Takada *et al.* showed, when rat kidneys are subjected to warm or cold ischemia the upregulation of E-selectin is low at 3-hour post-reperfusion and does not reach the peak until after 6 hours of reperfusion. Not a single construct disclosed in the Rittershaus *et al.*, if delivered by pre-transplantation perfusion, would retain in an ischemic human kidney long enough to exert any effect on complement activation because it would be washed out of the organ before its cellular target is expressed.

In contrast, the construct SCR(1-3), with the myristoylated peptide, can bind to any cell membrane and is not dependent on the presence of an upregulated protein for binding. This enables delivery and retention of very high levels of a complement regulatory molecule to an organ. Applicant herewith

enclose a recent publication to show that the retention of bulk APT070 has been demonstrated in human kidney (see, Pratt *et al.* J *Amer Pathol.* 163, 1457-63, 2003. Copy enclosed).

Applicants also note that Rittershaus *et al.* disclosed a CR1 fragment, which was modified by glycoform manipulation. Such modification is not possible for the SCR(1-3), because, the 3 SCRs lack a single N-linked glycosylation site from which a sialyl Le^x structure could be attached. Thus, even if the composition of Ritterhaus *et al.* were to be retained in ischemic organs, they could not be derived from the region of CR1 utilized in the instant invention. This is evident to an skilled artisan and to one who knows the CR1 structure.

Applicants believe that the Examiner, by looking at the sCR1 structure utilized by Ritterhaus *et al.*, believed that the sCR1 peptides of Ritterhaus *et al.* would inherently comprise the sCRs, the SEQ ID NO. 1, and the membrane binding elements of the instant invention. Applicants point out that the examiner has undertaken a *post hoc* rationalization of the art because the art does not contain the teaching alleged by the examiner. Thus, Ritterhaus *et al.* do not anticipate the claimed invention. Therefore, applicants request withdrawal of the rejection.

On page 3 of the office action, the examiner rejected claims 9, 14, 15, 17, and 18 as being anticipated by Smith *et al.* (US Patent No. 6,713,606). The examiner alleged that the CR1 of Smith *et al.* "would comprise SCRs (claim 15) and membrane binding elements consistent with claim 17 [and] derivatized with an myristoyl group ([ ] claim 18)." Applicants respectfully disagree with the examiner. In response, in order to assist the examiner in distinguishing the claimed invention and the cited art, applicants submit the following:

Smith *et al.* disclose complement regulators modified with combinations of membrane-binding elements that can be administered to patients. This

usefulness results from the effect of complement regulators such as CR1, in inhibiting the generation of neutrophil chemotactic agents, and particularly C5a. Hence, reducing the neutrophil-mediated post-reperfusion damage as well as the damage mediated directly by the cytolytic effects of the complement system. The examiner has not addressed the unexpected results obtained by using the claimed soluble derivative APT070 (claim 9 is now amended to recite the compound name APT070, for clarity). Applicants herewith submit a recent publication to show the unexpected finding of the effect of APT070 on the cellular immune system (see Pratt *et al.* 2003, for example, Figure 6). The results show that pre-treating kidneys with APT070 by perfusion led to a reduction of staining for CD3 and CD45 antigens in transplanted kidneys, which is an indication of reduced infiltration by T-cells (see Pratt *et al.*, Fig 6f). This means that treatment with APT070 in some way reduced the immunogenicity of the grafted organ - a very important influence on the subsequent fate of the graft. Pratt *et al.* concluded that "the transplanted kidney may be a particularly important organ for therapeutic complement regulation." This conclusion emerged from studies of animals deficient in decay-accelerating factor (DAF, CD55). CR1 also has this activity but it is localized specifically in certain regions of the multi-sCR structure of CR1. The fragment of CR1 used in APT070 possesses strong DAF-like activity but other recombinant fragments of CR1 do not. Thus, the T-cell immunomodulatory effects of APT070 are due to a combination of a type of regulatory activity found within this specific CR1 fragment and a membrane-binding element array which together permit the agent to function like exogenous DAF.

The above described immunomodulatory mechanism could not have been predicted from the membrane-localizing concept disclosed in Smith *et al.*, nor by the mechanisms of cellular adherence exploited by Ritterhaus *et al.* Its significance lies in the combination of known anti-reperfusion injury mechanisms

and immunomodulatory function which makes the soluble derivative APT070 particularly well suited for application in transplantation.

As noted above, applicants amend claim 9 for clarity and to further define that the recited soluble derivative is APT070. Therefore, Smith *et al.* do not anticipate the claimed invention.

Withdrawal of the anticipation rejection is requested.

Obviousness Rejection:

On pages 3-4 of the office action, the examiner has rejected claims 9 and 18 under 35 USC § 103(a) and alleged as being unpatentable over Ritterhaus *et al.* in view of Smith *et al.* The examiner states that sCR1 peptides of Ritterhaus *et al.* would inherently comprise the sCRs, the SEQ ID NO. 1, and the membrane binding elements of the instant invention. The examiner further speculates that a skilled artisan would be able to combine the sCR1 peptides of Ritterhaus *et al.* and the myristoyl group of Smith *et al.* to arrive at the composition used in the claimed methods. Applicants respectfully disagree with the examiner and refer to arguments above, made in response to the alleged anticipation rejection.

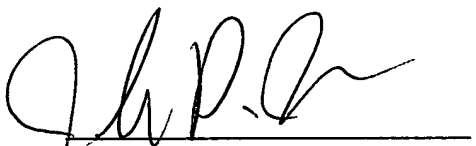
Applicants also point out that Smith *et al.* do not disclose the method for preparing an organ using the soluble derivative APT070 composition (amended claim 9 now recite APT070, for clarity) and do not rectify the deficiencies of Ritterhaus *et al.*, as described above. Therefore, the combination of Smith *et al.* and Ritterhaus *et al.* does not make the claimed invention obvious.

Thus, withdrawal of the obviousness rejection is requested.

REQUEST

Applicants submit that the claims are in condition for allowance, and respectfully request favorable consideration to that effect. The examiner is invited to contact the undersigned at (202) 912-2000 should there be any questions.

Respectfully submitted,



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American Journal of Pathology, Vol. 163, No. 4, October 2003

Nontransgenic Hyperexpression of a Complement Regulator in Donor Kidney Modulates Transplant Ischemia /Reperfusion Damage, Acute Rejection, and Chronic Nephropathy

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From the Department of Renal Medicine and Transplantation, King's College, University of London, London; and Adprotech Limted,† Essex, United Kingdom*

Complement activation during ischemia and reperfusion contributes to the development of tissue injury with severe negative impact on outcomes in transplantation. To counter the effect of complement, we present a strategy to deliver a novel complement regulator stabilized on cell surfaces within donor organs. The membrane-bound complement regulator is able to inhibit complement activation when the donor organ is revascularized and exposed to host-circulating complement. Application of this construct to donor kidneys protected transplanted tissues from ischemia/reperfusion injury and reduced the deposition of activated complement and histological signs of damage under conditions in which a nontargeted control construct was ineffective. Treatment of donor organs in this way improved graft performance in the short and long term. An analysis of the immune response in allograft recipients showed that reducing graft damage at the time of transplantation through complement regulation also modulated the alloresponse. Additionally, the results of perfusion studies with human kidneys demonstrated the feasibility of targeting endothelial and epithelial surfaces with this construct, to allow investigation in clinical transplantation. ***(Am J Pathol 2003, 163:1457–1465)***

Ischemia/reperfusion (I/R) injury is inherent in transplantation because every donor organ by definition is devoid of blood for a period of time. The tissue damage caused is in part dependent on the length of ischemia and also mediated by complement.[1] This has been shown for several models of ischemia, for example in the gut, kidney, and skeletal muscle, using both complement-deficient mice and complement-inhibiting agents.[2–4]

To address the difficulties of preventing I/R damage, we have treated donor organs with a novel, modified membrane-binding complement regulator. Our approach is designed to prevent complement activation specifically within a transplanted organ. The ligands for complement regulators are strictly the active fragments of complement components present only at the site of complement activation. It is now recognized that the kidney itself is a significant source of complement components.[5] Recent experimental work has suggested that such local synthesis of complement has great influence on local tissue injury in the transplanted kidney[6] and that local production in the kidney, for example of the pivotal component C3, which links the activation and terminal cascades of complement, is increased in response to I/R injury.[7] In addition, ischemia/reperfusion injury and its contributory factors have a serious negative impact on acute rejection and the long-term results in clinical transplantation.[8]

We have modified a form of human complement receptor type 1 (CR1, CD35) as a means to reduce I/R injury in transplanted kidneys. Human CR1 is found on the surface of almost all peripheral blood cells, but has a limited distribution on other cell types. It is, however, a potent inhibitor of complement activation, having decay accelerating activity for the disassembly of the complexed C3 convertases of the classical and alternative activation pathways, and co-factor activity for Factor I, which inactivates C3b (Figure 1a).[9] The active site in CR1 was initially reported to reside in the first four short consensus repeats (SCRs) in each long homologous repeat[10] but is probably bounded by the first three domains—at least in long homologous repeat (LHR)-A.[11] Each of the functions of CR1 have been identified in other complement control proteins, but the presence of multiple functions in CR1 makes it an ideal candidate for therapeutic tissue engineering.[12]

To confer ability to localize the soluble SCR1-3 fragment on target cell surfaces, a membrane-binding moiety

Supported by the Guy's and St. Thomas' Kidney Patients Association (to J. P.) and Adprotech Ltd.

J. R. P. and M. E. J. contributed equally to the study.

Accepted for publication June 25, 2003.

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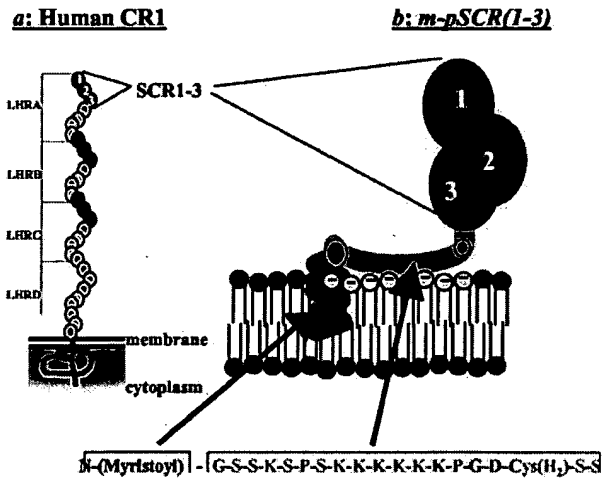

Figure 1. Comparative structure of human CR1 and m-pSCR1-3. **a:** Human CR1 exists on the cell surface as a string of short consensus repeat (SCR) domains attached via a transmembrane portion to a short cytoplasmic domain, each SCR being of 60 to 70 amino acids. Several allotypes exist, differing in the number of SCRs. The most common form, allotype A, consists of 30 SCRs and exhibits a further level of internal homology in the form of long homologous repeats (LHRs) of every seven SCRs totaling 235 kd. **b:** m-pSCR1-3 consists of recombinant SCR(1-3) of LHR-A of CR1 attached to the membrane-binding tag. The tag consists of a myristoyl tail chemically linked to a synthetic positively charged peptide whose terminal cysteine enables attachment of the SCR(1-3) of CR1. This water-soluble molecule is ~23 kd in size and retains the known complement regulatory mechanisms of intact CR1 as well as the ability to bind cells. The construct is designed to insert itself into the hydrophobic core of the lipid bilayer of cell membranes and binding is stabilized by the basic peptide region allowing presentation of the functional moiety, in this instance human SCR(1-3) of CR1.

was designed for posttranslational chemical attachment to SCR1-3 (Figure 1b).[13,14] This was based on a linear array of membrane-binding units, each of a different specificity and assembled into a synthetic peptide termed MSWP1[14] based on the myristoyl electrostatic switch[15] for membrane localization. The myristoylated peptide membrane-binding product of this process, termed m-pSCR1-3 (Figure 1b), is a soluble, extrinsically administered protein with membrane-binding properties capable of intrinsically regulating complement activation on cell surfaces and able to exert a local and persistent anti-inflammatory effect.[16]

Here we report the ability of the membrane-targeted complement regulator to bind to rat and human kidney, and to reduce antigen-independent and antigen-dependent injury of rat donor kidney in subsequent transplantation studies.

Materials and Methods

Design of a Recombinant Low-Molecular Weight Complement Regulator

The gene coding for the N-terminal SCR1-3 of LHR-A of CR1 with a codon for an additional C-terminal cysteine was constructed as described by Dodd and colleagues,[17] and a plasmid, pDB1081–1 constructed as described in Mossakowska and colleagues[11] for transformation into the expression host *Escherichia coli* BL21 (DE3). The protein product of the expression vector was purified and shown to inhibit antibody-sensitized red blood cell lysis by human serum, to inhibit C3a release, and to function as a co-factor for Factor I.[17] The analysis of activity revealed that the 21-kd SCR1-3 fragment was active against both the classical and alternative pathways and appeared to be correctly folded.[11,13,17]

Membrane-Binding Reagents

The membrane-localizing thiol-reactive agent *N*-(myristoyl)-Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-(*S*-2-thiopyridyl)-Cys-carboxamide was prepared by solid-phase synthesis as described previously.[11] The corresponding reduced (free cysteine) form lacking SCR1-3 and used as a control agent in this study was designated "m-p" (myristoylated peptide).

Construction of a Membrane-Binding Complement Regulatory Molecule, m-pSCR(1-3)

A plasmid encoding SCR1-3cys of CR1 was constructed and expressed in *E. coli* BL21 (DE3) as described by Mossakowska and Smith,[12] purified by the method of Dodd and colleagues,[17] coupled to the membrane-binding tag m-p, and isolated as described.[13] The product had M_r ~24 kd by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a 50% inhibitory concentration in a classical pathway hemolytic assay of ~0.15 nmol/L. using a commercially available antibody-sensitized sheep red blood cell preparation (Diamedix, Miami, FL) and a dilution of human serum of 1:400.[11]

Kidney Transplantation and Treatment with m-pSCR(1-3)

Animals were purchased from Harlan Olac (Bicester, UK) and maintained with standard rat chow and water *ad libitum*. All animal experiments were conducted within the restrictions of a Home Office License for animal experimentation. Anesthesia was performed with isoflurane administered through a vaporizer. The left donor kidney was dissected and the aorta ligated cephalad and caudad to the renal artery. A portex tube catheter (0.5-mm outside diameter) (Portex Ltd, Hythe, UK) was inserted between the ligatures and the kidney was perfused with 5 ml of Soltran Kidney Perfusion Solution (Baxter Health Care, UK) containing 200 μg of m-pSCR1-3 (40 μg/ml) throughout a period of 5 minutes. The kidney was then placed in ice-cold saline for 30 minutes. Orthotopic renal transplantation was performed using the method of Fabre and colleagues[18] and microvascular clips releasing the blood flow were removed once 60 minutes of warm ischemia had elapsed. Syngeneic transplantation was performed using DA rats. The 7-day experiment used groups of six rats, and the 20-week experiment used groups of five and six control and treated rats, respectively. The allograft experiment with Fischer F344 ($RT1^{lv1}$) rat donors and

Lewis ($RT1^l$) rat recipients used groups of six control and seven treated rats.

Analysis of Transplant Recipients

At regular intervals after transplantation blood samples were collected for analysis of blood urea nitrogen as previously described.[19] Kidney transplants were harvested at various time intervals and portions of tissue were placed in 4% formal saline for standard histological analysis, or frozen in liquid nitrogen for subsequent immunohistochemical staining, using antibodies against C3d (DAKO, Glostrup, Denmark), C5b-9 (Quidel, CA), CD3 (Becton Dickinson, Abingdon, UK), and CD45 (Becton Dickinson, Abingdon, UK), and for morphometric image analysis as previously described.[20]

Analysis of m-pSCR(1-3) Binding to Rat Kidney Cells

Immortalized rat renal endothelial cells[21] or primary cultured rat tubular epithelial cells[7] were incubated with varying concentrations of m-pSCR1-3 in phosphate-buffered saline (PBS) for 40 minutes at 4°C. For immunocytochemistry cells were grown on glass coverslips and for flow cytometry cells were detached from plastic culture flasks and aliquots of 5×10^5 cells exposed to m-pSCR(1-3). For immunocytochemistry cells were washed twice in PBS and then fixed in cold acetone and stained using Cy3-labeled 3E10 [mouse anti-human SCR1-3 monoclonal antibody (mAb); provided by Adprotech Ltd.] for 30 minutes at 4°C, mounted in Fluoromount (Sigma), and examined using a fluorescence microscope.

For flow cytometry, cells were washed twice in PBS and then incubated with Cy3-labeled 3E10 for 30 minutes at 4°C, washed twice, and fixed in 1% paraformaldehyde in PBS. The samples were analyzed using a FACscan flow cytometer (Becton Dickinson).

Mixed Lymphocyte Reaction

Responder and stimulator lymphocytes were prepared as previously described.[6] Cellular proliferation was assessed using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). This is a colorimetric method in which methyl tetrazolium salt (MTS) is reduced into a soluble formazan product. The absorbance at 490 nm provides an index of the number of viable proliferating cells, which was determined at days 1, 3, 5, and 7 of the assay.

Perfusion of Human Tumor Nephrectomy Specimens

Human kidneys were obtained, with research ethics committee approval and patient consent, from patients undergoing nephrectomy for the removal of renal cell carcinoma in which there was a defined tumor mass recognized by preoperative imaging. Organs were placed on ice and perfused via the renal artery with 1 L of ice-cold Soltran solution with ($n = 4$) or without ($n = 1$) m-pSCR1-3 at 80 μg/ml. Tissue samples were taken at frequent time intervals (approximately every 2 hours) for 24 hours, and processed for histological analysis, for immunohistochemistry, and by enzyme-linked immunosorbent assay to determine the presence of m-pSCR(1-3).

Statistics

Student's t-test or repeated measures analysis of variance was used where appropriate to determine significance between treatment groups; $P < 0.05$ was taken as significant.

Results

m-pSCR(1-3) Binds to Rat Kidney Endothelial and Epithelial Cells

We incubated cultured rat glomerular endothelial cells with m-pSCR1-3 and assessed binding by immunocytochemistry using a labeled mouse anti-human SCR1-3 mAb (3E10) (Figure 2, a and b). We found that m-pSCR(1-3), but not the untagged SCR1-3 bound in a dose-dependent manner (Figure 2, c and d). Binding of m-pSCR1-3 also occurred on cultured rat tubular epithelial cells (data not shown). In a hemolytic assay using antibody-sensitized sheep erythrocytes, the addition of the m-p membrane-binding tag to SCR1-3 produced a substantial (~100-fold) increase in inhibitory activity compared to untagged SCR1-3 (unpublished data).[13]

m-pSCR(1-3) Reduces Acute Complement-Mediated I/R Injury

The routine flushing of donor kidneys with perfusion fluid before transplantation presents an ideal opportunity to introduce m-pSCR1-3 into donor organs. We used this approach in a rat model of syngeneic renal transplantation. We perfused the donor graft with ice-cold kidney perfusion solution containing either m-pSCR1-3 or a control substance, before exposing the donor tissue to 30 minutes of cold ischemia and 60 minutes of warm ischemia. This schedule was chosen because it produced histological and functional damage followed by partial recovery in untreated transplants.

The distribution of m-pSCR1-3 in the donor kidney is shown in Figure 3; a to c. The pattern of staining was consistent with binding of m-pSCR1-3 to the glomerular capillary and peritubular capillary walls and the tubular epithelium at its basal surface. Histological analysis at 24 hours after transplantation showed reduced signs of acute tubular necrosis (tubular attenuation and cellular swelling, and associated neutrophil infiltration) in m-pSCR1-3-treated grafts (Figure 3, d and e). At 24 hours after transplantation tissue myeloperoxidase activity, taken here as a measure of neutrophil activity, was sig-

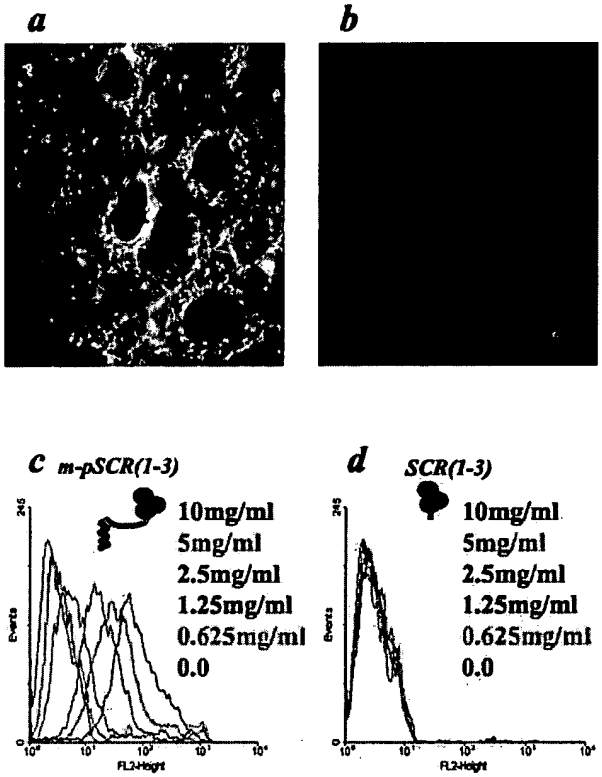

Figure 2. Membrane binding of m-pSCR(1-3). **a:** m-pSCR1-3 was detected using Cy3-labeled anti-SCR(1-3) mAb (3E10) on cultured transformed rat renal endothelial cells exposed to 2.5 μg/ml of m-pSCR1-3. The dome of the cell is outside the focal plain of the photomicrograph giving the appearance of a central dark area. **b:** Staining was not present on endothelial cells incubated with SCR1-3 lacking the m-p tail. **c:** Dose-dependent cell-surface binding to rat endothelial cells *in vitro* detected by FACS in the presence of the membrane-binding tag. **d:** No binding occurred at any concentration of SCR(1-3). Original magnification, ×1000 (**a**).

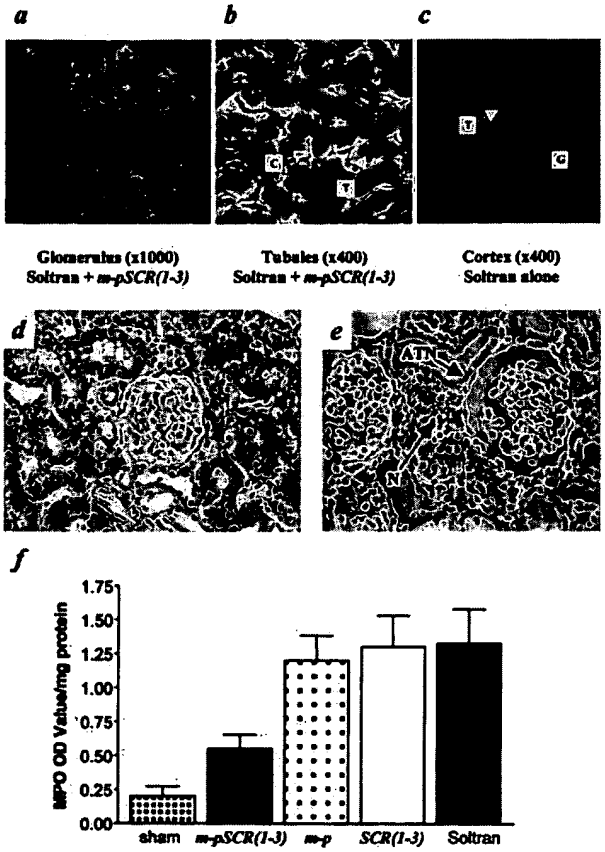

Figure 3. Immunohistopathology of perfused donor kidneys Isolated rat kidneys were flushed with 5 ml of kidney perfusion solution containing 200 μg of m-pSCR(1-3). The tissue distribution of m-pSCR1-3 is shown after immunofluorescent staining with Cy3-labeled 3E10. **a:** Glomerulus with capillary loops stained for m-pSCR(1-3). **b:** Basolateral surfaces of tubules (T) and peritubular capillary wall (C) outlined by the staining for m-pSCR(1-3). **c:** The cortex of a control kidney perfused with carrier solution alone and stained with Cy3-labeled 3E10. Glomerulus (G) and tubules (T) are negative. H&E staining of illustrative donor kidney sections 24 hours after transplantation showed well-preserved architecture if perfused with m-pSCR1-3 (**d**) whereas carrier-only perfused kidneys (**e**) showed marked tubular flattening (ATN) and some infiltration of neutrophils (N). Tissue myeloperoxidase activity 24 hours after transplantation (**f**) showed significantly reduced MPO activity in m-pSCR1-3-treated grafts ▣ compared with control-treated grafts perfused only with carrier solution ■ ($P = 0.03$). MPO levels in SCR(1-3) □- and m-p ▨-treated grafts were not significantly different from controls. Sham-treated animals ▩ had a laparotomy 24 hours before MPO measurement ($n = 4$/group).

nificantly reduced in m-pSCR1-3-treated grafts compared to control grafts (Figure 3f). Deposition of the complement split product C3d (not shown) and the membrane attack complex C5b-9 was absent or weak in m-pSCR1-3-treated grafts, whereas staining was more intense and widespread in control-treated grafts (Figure 4). These results indicate that m-pSCR1-3 treatment reduced the cleavage of C3 and the subsequent formation of C3d and C5b-9. The amount of tissue-bound m-pSCR1-3 detected by immunohistochemistry declined after 24 hours, so that on day 7 it was undetected in treated tissues.

Renal function in recipients of m-pSCR1-3-treated grafts was significantly better throughout the first week after transplantation compared to controls (Figure 5a). Among control grafts, including those treated with the untagged complement regulator, SCR(1-3), there was no significant difference in function. Therefore both functional and structural improvement required the inhibitor to be bound to treated cell surfaces via the m-p moiety.

m-pSCR(1-3) Treatment of Donor Organs Improves Long-Term Graft Function

We next investigated whether reducing the acute complement-mediated I/R injury could improve the long-term outcome of isografts. This is important because early graft injury may be a driver of late graft failure.[22] In this experiment we followed m-pSCR1-3- or control-treated ischemic isografts for up to 20 weeks after transplantation. We found that m-pSCR1-3 treatment reduced the degree of functional (Figure 5b) and histological graft injury throughout the period of study, such that by 20 weeks m-pSCR1-3-treated grafts showed only mild features of cellular swelling (Figure 5, c and e), whereas control grafts demonstrated widespread swelling and intimal proliferation of the arteriolar walls (Figure 5, d and f). Thus, transient initial treatment of the donor organ had a durable and beneficial effect.

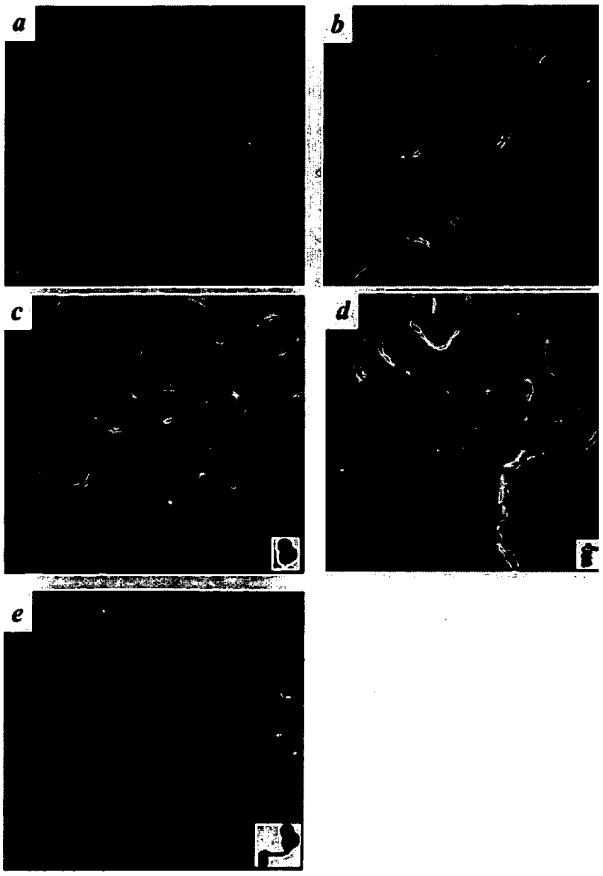

Figure 4. Membrane attack complex formation at day 1 after transplantation. Immunohistochemistry for C5b-9 membrane attack complex showing a normal, sham-operated kidney (**a**), a graft treated with perfusion solution alone (**b**), effect of treatment with perfusion solution containing untagged SCR(1-3) (**c**), effect of treatment with the m-p tag alone (**d**), and effect of treatment with m-pSCR(1-3) (**e**). Control-treated grafts undergoing ischemia and reperfusion exhibited widespread tubular deposition of C5b-9. Grafts protected by m-pSCR1-3 showed markedly reduced deposition of C5b-9. Original magnifications, ×1000.

m-pSCR(1-3) Treatment of Donor Kidney Reduces Allograft Rejection

To examine the effect of reduced I/R injury on the immunogenicity of the graft, we used the well-described Fischer F344 ($RT1^{v1}$) to Lewis ($RT1^{l}$) renal allograft model.[23] The donor and recipient differ by a single MHC class I allele, resulting in a mild and spontaneously resolving acute rejection episode that peaks between 10 and 14 days after transplantation. The protocol for inducing graft ischemia and treatment with complement inhibitor or control solution was the same as before. On day 14 after transplantation, histological damage (Figure 6; a to d) and renal dysfunction (Figure 6e) in allografts treated with m-pSCR1-3 were reduced. This protective effect of m-pSCR1-3 was evident throughout the period of acute rejection.

To measure the degree of leukocyte infiltration, we performed immunohistological examination of common leukocyte (CD45) and specific T-cell (CD3) markers. Image analysis of the stained tissues at the peak rejection time indicated there was a reduction of infiltration by total leukocytes and T cells in m-pSCR1-3-treated grafts (Figure 6f).

In addition we examined the anti-donor T cell proliferative response of the recipient on day 14 after transplantation. The T-cell response in recipients of m-pSCR1-3-treated grafts was reduced in comparison with recipients of control grafts (Figure 6g), indicating that the treatment of donor organs with the tagged complement regulator led to modulation of recipient T-cell stimulation.

m-pSCR(1-3) Binds to ex Vivo *Perfused Human Kidney*

To assess the feasibility of treating human organs, we obtained human tumor nephrectomy specimens in which the whole kidney had been removed on a short length of renal artery. We then perfused the organ with a solution containing m-pSCR1-3 and examined the distribution of m-pSCR1-3 in the nontumor tissue. Immunohistochemical analysis showed that m-pSCR1-3 localized, as in the rat studies, to the glomerular and tubular structures of the kidney (Figure 7, a and b). The presence of m-pSCR1-3 in blocks of tissue was confirmed by enzyme-linked immunosorbent assay (data not shown). Although the level of inhibitor detected by enzyme-linked immunosorbent assay was variable, suggesting that perfusion or tissue binding was not equal throughout the kidney, the inhibitor was detected in all areas sampled.

Discussion

Although there is strong circumstantial evidence that graft prognosis is influenced by the severity of donor organ injury sustained during the transplant procedure,[24] to date there have been few experimental reports to support this concept, much less a successful therapeutic strategy that prevents organ damage at the time of transplantation. We have developed a novel strategy to inhibit complement activation within donor organs used for transplantation. Our approach to treat donor kidney with membrane-targeted complement regulator had two important effects. Firstly, treatment increased the resistance of the donor kidney to acute I/R injury, which led to better long-term graft performance and reduced chronic damage. Secondly, the prevention of acute complement-mediated I/R injury lessened the immunogenicity of the graft in nonidentical transplant recipients. Our findings support the general notion that the inflammatory condition of the graft is a driver of antigen-independent and antigen-dependent injury. Moreover, they suggest that therapeutic manipulation of the donor organ is an effective means to prevent such injury.

I/R injury in clinical practice manifests most acutely as delayed graft function and may permanently decrease the function of transplanted organs. Increased morbidity and mortality associated with delayed graft function have been noted in clinical studies and represent a relationship between the condition of the engrafted tissue and the subsequent host response.[25,26] In European studies

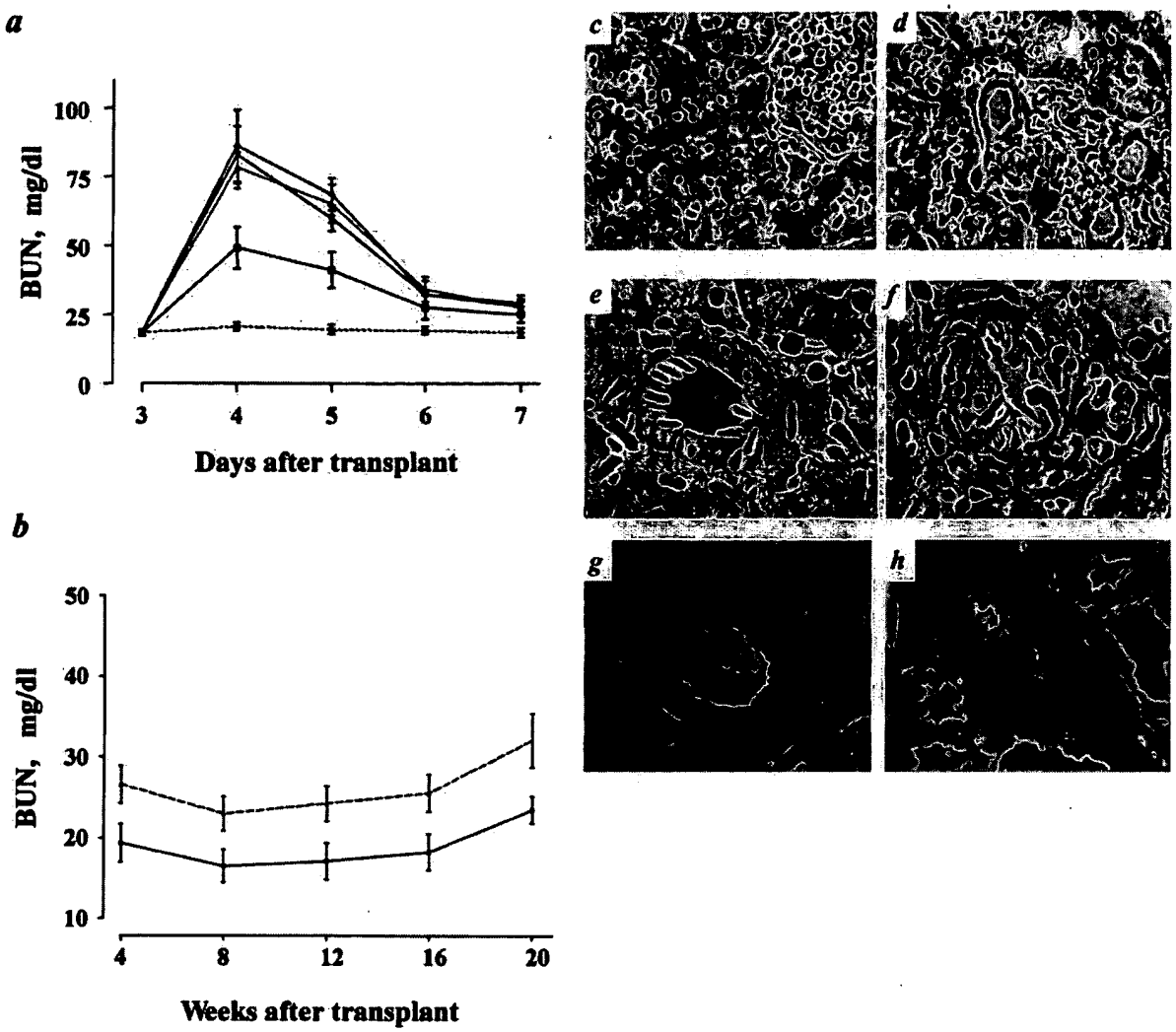

Figure 5. Functional and histological analysis of renal isografts. **a:** Blood urea nitrogen up to 7 days after transplantation in recipients of grafts treated with m-pSCR1-3 —■—, perfusion solution only —◆—, SCR(1-3) lacking the m-p tail —▲—, or the m-p tag alone —▼— or in sham-operated rats undergoing nephrectomy without transplantation ···●···. Data are shown from day 3 because this was the point from which renal function was dependent on the transplanted kidney alone. Treatment with m-pSCR1-3 led to significantly improved renal function compared to any one of the control groups ($P < 0.001$; $n = 6$ animals/group). **b:** Blood urea nitrogen throughout 20 weeks after transplantation after perfusion of donor graft with perfusion solution alone –▲–, or m-pSCR1-3 —●—. Protection conferred by m-pSCR1-3 in the first week led to improved function throughout 20 weeks ($P < 0.001$; $n = 6$ m-pSCR1-3-treated grafts and $n = 5$ perfusion solution-treated controls). H&E staining of illustrative donor kidney sections showed well-preserved tubular structure in m-pSCR1-3-treated grafts at 20 weeks (**c**) compared to carrier-treated controls (**d**). Vessels also had a near normal appearance in the m-pSCR(1-3)-treated grafts (**e**), compared to carrier-treated grafts at 20 weeks in which arteriolar intimal proliferation was present (**f**). No elastic duplication was seen in untreated (**g**) or treated grafts (**h**). Original magnifications: ×400 (**c**, **d**); ×1000 (**e**, **f**).

delayed graft function affects as many as 20 to 30% of cadaveric renal transplants.[27] Moreover, the increasing discrepancy between numbers of patients listed for transplantation and potential donors has necessitated the use of so-called suboptimal donors, ie, older than 50 years old and/or organs that have experienced more than 24 hours of cold ischemia. At present no specific agent is used to reduce the susceptibility of transplanted organs to I/R injury, the risks and implications of which increase significantly with suboptimal donor organs.[27]

Within minutes of I/R injury, activation and deposition of complement occurs, with subsequent up-regulation of cytokines and adhesion molecules[28] that mediate the infiltration of leukocytes. Leukocyte accumulation and adhesion to microvascular endothelium in postischemic tissues is thought to be important through the release of toxic oxygen radicals and inflammatory cytokines.[29] Postischemic complement activation is thought to cause direct endothelial and epithelial tissue damage[30] and C5a-mediated neutrophil chemotaxis,[31] which were reduced in the m-pSCR1-3-treated grafts in our study.

Soluble forms of naturally occurring complement regulators have been assessed in models of I/R injury.[2–4] The soluble inhibitors currently available have been given systemically and have relatively short functional half-lives. This has necessitated the use of high doses that systemically abrogate the function of the complement system and that have the potential to lower host antimicrobial defenses. This is potentially undesirable for transplant recipients receiving other immunosuppressive

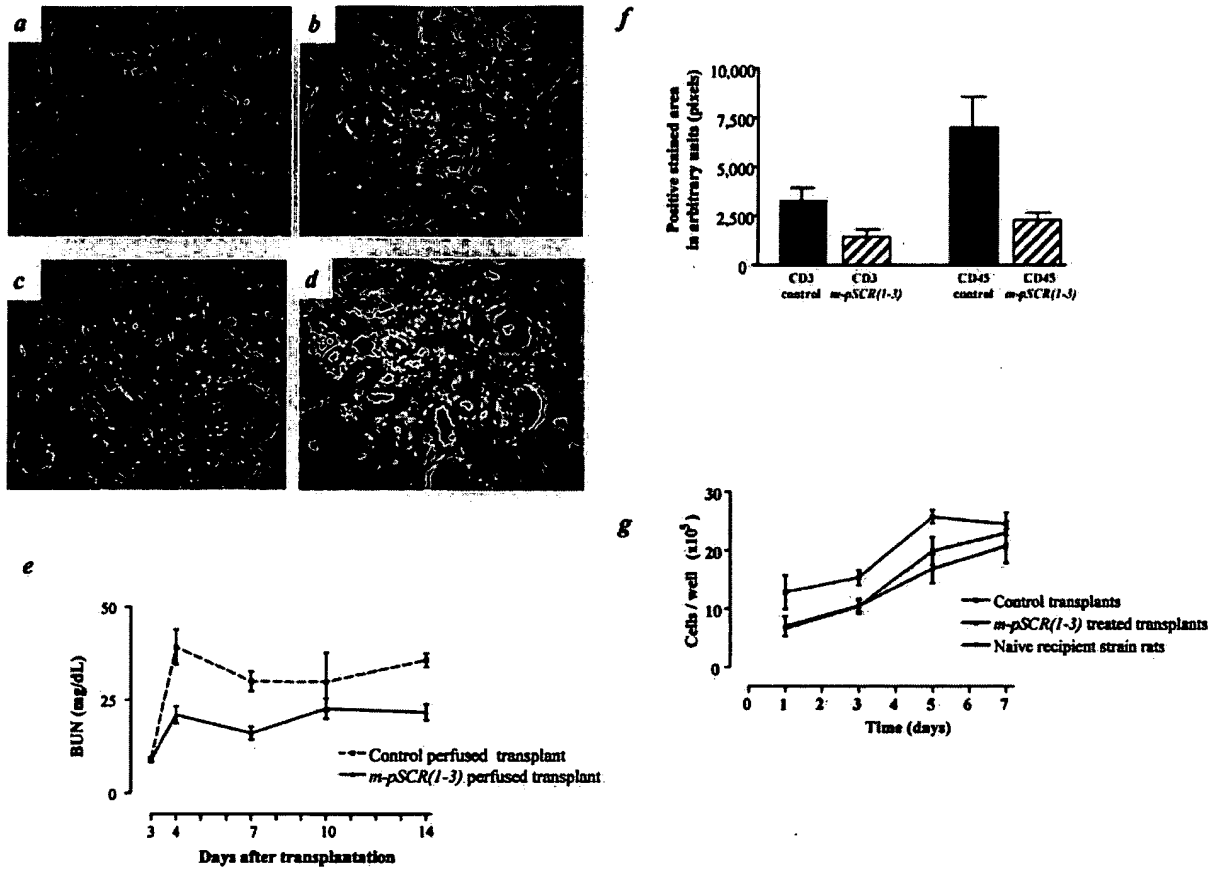

Figure 6. Effects of m-pSCR1-3 on renal allografts. Histological analysis of F344 to Lewis renal allografts at day 14 after transplant showed signs of cellular infiltration in all transplants. However, the degree of interstitial infiltration of mononuclear cells and evidence for tubulitis was less in m-pSCR1-3-treated grafts (**a** and **b**) compared to control transplants treated with perfusion solution only (**c** and **d**; n = 8/group). The m-pSCR1-3-treated grafts also showed significantly improved renal function compared to perfusion solution-treated controls (**e**) between days 4 and 7 ($P < 0.0001$), which was 24 hours after removal of the remaining native kidney. BUN levels <10 mg/dL were taken to be within normal range (for animals with two functioning kidneys). Morphometric analysis of antibody-stained frozen sections (**f**) showed reduced CD45 and CD3 staining in m-pSCR1-3-treated kidneys at day 14 after transplantation, compared to controls treated with perfusion solution only. Data are expressed in arbitrary units (pixels) and derived from 15 representative cortical fields from three transplanted kidneys in each group. Proliferation of spleen T cells isolated from transplant recipients at day 14 after transplantation (**g**) showed significantly reduced alloresponse in the recipients of m-pSCR1-3-treated kidneys compared to recipients of control-treated kidneys, analyzed throughout 7 days by MTS assay (n = 4 animals/group assayed in triplicate, in comparing the treatment group with either control group, $P < 0.01$). Original magnifications, ×250 (**a–d**).

drugs. Attempts have been made to prolong the half-life of such reagents, for example by linking complement regulators to albumin,[32] or to sialyl Lewis-x moieties to target endothelial surfaces.[33] The coupling of complement regulators to Ig domains has also been shown to prolong half-life,[34] but not to protect the kidney from I/R injury.[35] In our study, the untagged form of SCR(1-3) of CR1 similarly did not prevent I/R injury suggesting that the complement regulator must be bound to the target of complement activation at the site of tissue injury for it to be effective. Our therapeutic targeting strategy has overcome these difficulties.

We have addressed two issues in the targeting of therapeutic complement regulators. Firstly we have considered where in the complement cascade therapeutic intervention is effective. Secondly, we have determined how to target complement inhibition to those tissues that bear damage. By using CR1 activity as the basis for intervention, we focused our approach on the inactivation of C3b, because this is the pivotal bridging step between the activation and terminal pathways. Recent studies of I/R in C6-deficient mice have highlighted the importance of direct membrane damage caused by the membrane attack complex,[36] while the upstream release of the fragments C3a and C5a, which have anaphylactic and chemotactic properties, contributes to local inflammation. The CR1-derived molecule used in our novel construct is a structurally minimal component, retaining the activities of CR1 needed for breakdown of complexed C3b within C3 convertases, and thus for its inhibitory effect on the generation of C3a, C5a, and membrane attack complex.[11] The design of the membrane-binding tail exploits the membrane-inserting property of myristate, in combination with an electrostatic contribution to binding from a synthetic basic peptide sequence that interacts with negatively charged phospholipid headgroups, enabling binding to the cell surface. Combined with routine perfusion of the donor organ, this would permit organ-specific delivery and retention of m-pSCR(1-3). This two-site membrane interaction used in the targeting peptide exploits additive binding affinities of small molecules for spatially related components of cellular membranes[14]

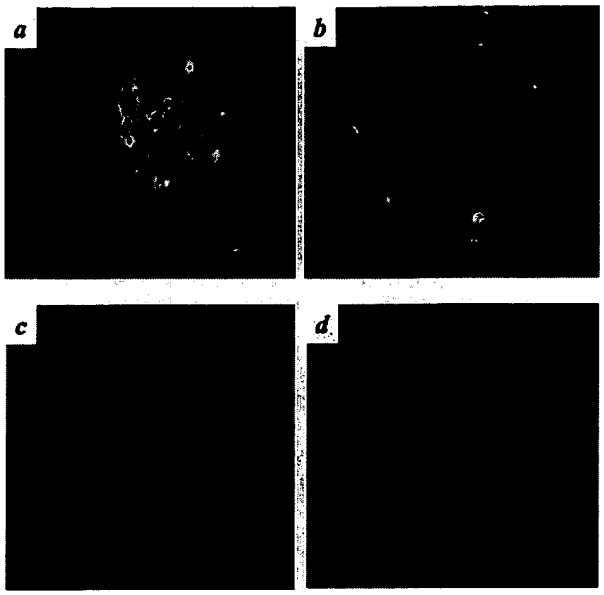

Figure 7. Binding of m-pSCR1-3 to human kidney. Immunohistochemistry of human kidneys perfused with preservation solution containing m-pSCR1-3 showed (**a** and **b**) that the agent bound to glomeruli (G) and to tubules (Tu). **c:** The high-power view reveals a pattern of m-pSCR1-3 staining consistent with binding to the basolateral tubule surface (B). **d:** Human kidney perfused without m-pSCR1-3 showed no staining. Original magnifications: ×1000 (**a**, **b**, **d**); ×2000 (**c**).

and allows flexibility because the m-p tails can be modified to suit different applications. Site-directed regulators of complement activation have a wide variety of clinical applications beyond transplantation[14,16] and this type of membrane localization strategy is also of wide applicability.

Our results show a significant antigen-independent component of injury late after transplantation that was preventable by modifying acute I/R injury. Additionally, there was an effect on the antigen-specific component of injury, suggested by the finding of reduced alloreactivity in the recipients of m-pSCR1-3-treated grafts. Thus the overall potential to improve graft function could reside in the benefit of reduced immunogenicity of the graft as well as reduced postischemic damage. A clinical corollary may be found in the results of live unrelated organ transplantation, in which ischemic times are minimized and consequently clinical outcomes are improved compared to cadaveric renal transplantation with equivalent degrees of MHC mismatch.[37]

Complement activation is possibly one of a number of antigen-independent mechanisms that enhance the recognition of foreign tissue by the recipient immune system. Current debate on the role of nonspecific injury in the stimulation of T-cell immunity would suggest that reduced inflammation could attenuate specific immunity.[38] The results presented here support this concept, and suggest that intragraft inhibition of complement decreases the local inflammatory environment and reduces the formation of C3 fragments with immunoregulatory properties. Indeed, recent experiments using a mouse kidney allograft model found that donor organs with reduced capacity for the production of C3 elicit weaker alloimmunity,[6] suggesting an important effect of local complement. Thus the transplanted kidney may be a particularly important target for therapeutic complement regulation.

To maximize the efficacy of therapeutic complement regulation, we introduced the complement regulator before complement activation, that is, before reperfusion of the transplant organ with recipient blood. By adding m-pSCR1-3 to the perfusion solution we were able to intervene in the pathogenesis of I/R injury. This method bypassed the inherent time delay for DNA transcription and protein translation required using gene transfection technologies to introduce site-specific protein expression. In organ transplantation this is particularly relevant because of the lack of notice clinicians receive before an organ transplant. The degree of protection we observed was achieved with a single application of a molecule that has been shown to be acceptable for use in humans.[20]

In conclusion, we have shown that it is possible to modify the biological properties of donor kidney by targeting a complement regulatory protein to potential sites of tissue injury, thereby reducing the late consequences of inflammatory and immunological injury. The approach described here in a rat model has the potential to be applied to clinical evaluation, possibly in conjunction with other emerging therapies for transplant I/R damage, such as superoxide dismutase mimetics[39] and adhesion molecule blockade.[40] Transient modification of the donor organ without the need for systemic complement inactivation might have a significant advantage for kidney transplant recipients.

Acknowledgments

We thank Mr. Rodney Oldroyd, Mr. Ian Dodd, and members of the Development Team at Adprotech for their assistance and helpful discussions; and Dr. Louise Affleck for helping us with antibody reagents.

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The Cytokine-adhesion Molecule Cascade in Ischemia/Reperfusion Injury of the Rat Kidney

Inhibition by a Soluble P-selectin Ligand

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Abstract

Ischemia/reperfusion (I/R) injury associated with renal transplantation may influence both early graft function and late changes. The initial (≤ 7 d) events of warm and in situ perfused cold ischemia of native kidneys in uninephrectomized rats were examined. mRNA expression of the early adhesion molecule, E-selectin, peaked within 6 h; PMNs infiltrated in parallel. T cells and macrophages entered the injured kidney by 2–5 d; the associated upregulation of MHC class II antigen expression suggested increased immunogenicity of the organ. Th1 products (IL-2, TNFα, IFNγ) and macrophage-associated products (IL-1, IL-6, TGFβ) remained highly expressed after 2 d. To examine directly the effects of selectins in I/R injury, a soluble P-selectin glycoprotein ligand (sPSGL) was used. Ischemic kidneys were perfused in situ with 5 μg of sPSGL in UW solution; 50 μg was administered intravenously 3 h after reperfusion. E-selectin mRNA remained at baseline, leukocytes did not infiltrate the injured organs throughout the 7-d period, and their associated products were markedly inhibited. Class II expression did not increase. No renal dysfunction secondary to I/R occurred. The early changes of I/R injury may be prevented by treatment with soluble P- and E-selectin ligand. This may reduce subsequent host inflammatory responses after transplantation. (*J. Clin. Invest.* 1997. 99:2682–2690.)

Key words: P-selectin • E-selectin • cytokine • adhesion molecule • P-selectin glycoprotein ligand

Introduction

Impaired or delayed kidney function after engraftment is one of the most common sequelae of renal transplantation, occurring in as many as 50% of cadaver grafts in some series (1). As this complication is rare in living donor kidneys, regardless of histocompatibility differences, the condition has been thought to result from a variety of circumstances, including the state of the donor and the effects of warm or cold ischemia, storage, and reperfusion injury (2, 3). Although the majority of the renal changes are reversible, hospital stay is increased, administration of immunosuppression is more difficult, dialysis is prolonged, and cost is amplified. There is also evidence that the incidence of both acute rejection and diminished long-term graft survival is higher in such organs than in those which function immediately (4). It seems likely that the events surrounding initial ischemia/reperfusion (I/R)[1] may trigger upregulation of MHC antigens; the potentially increased graft immunogenicity may promote host cellular infiltration and expression of cell products (5). This nonspecific inflammatory response may interact with the cytokine-adhesion molecule cascade characteristic of immune injury.

The early dynamics after I/R injury which are potentially involved in subsequent graft changes have not been defined completely. This insult presumably causes elevation of P-selectin, which is rapidly translocated to endothelial cell surfaces within 5 min of revascularization of the organ, initiating steps leading to tethering of PMNs to the vascular intima (6). Local production of IL-1β and/or TNFα by these leukocytes induces P- and E-selectin expression on endothelium which continues the cascade of events which increase cell adherence and infiltration of the injured tissues (7, 8). In this study, the relationship between the induced expression of E-selectin and cellular and molecular kinetics has been examined. This molecule is associated with infiltration of PMNs which themselves may trigger the activities of other leukocyte populations and expression of their products. We have assessed the role of a soluble form of P-selectin glycoprotein ligand (sPSGL) in inhibiting selectin activity and subsequent events in the damaged organs. It appears that successful blockade of the earliest steps in leukocyte adhesion (i.e., rolling) is effective in diminishing I/R injury. This manipulation may reduce later changes which contribute potentially to subsequent host alloresponsiveness.

Methods

Animals. Inbred male Lewis (LEW) rats (Harlan Sprague-Dawley, Indianapolis, IN) of 250–300 g were used throughout the experiments.

Experimental design and operative technique. Three experimental models were examined. The initial events developing after cold I/R were examined in detail with particular reference to inflammatory molecules, cellular infiltration, and expression of associated products. Patterns occurring after warm ischemia were used for comparison.

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Received for publication 13 September 1996 and accepted in revised form 25 March 1997.

1. *Abbreviations used in this paper:* ICAM, intercellular adhesion molecule; I/R, ischemia/reperfusion; PSGL-1, P-selectin glycoprotein ligand-1; RANTES, regulated upon activation, normal T cell expressed and secreted; RT-PCR, reverse transcriptase-PCR; sPSGL, soluble form of P-selectin glycoprotein ligand-1; UW, University of Wisconsin solution.

J. Clin. Invest.

0021-9738/97/06/2682/09 $2.00
Volume 99, Number 11, June 1997, 2682–2690

The inhibitory effects of a soluble P-selectin ligand administered both in the cold perfusate and to the animal after reconstitution of the circulation were assessed. Uninjured kidneys in uninephrectomized rats acted as controls. Three animals in each group were examined for each time period.

The right kidney of normal LEW rats was removed and the left kidney was isolated. For the studies of warm ischemia, the left renal artery and vein were occluded for 45 min then released; the organs do not recover if the injury is prolonged to 60 or 90 min. The abdominal incision was closed and the animals returned to their cages. To produce cold ischemia, the aorta was occluded proximal and distal to the left renal artery, and the left renal vein clamped with a vascular clip near its junction with the inferior vena cava. The aorta was then catheterized with a silicone tube (Silicone Medical Grade Tubing, 0.02 inch inside diameter, 0.037 inch outside diameter; Baxter Scientific Products, Boston, MA) and a drainage vent made in the renal vein distal to the vascular clip. The isolated kidney was then perfused slowly (2–3 min) with 10 ml of iced perfusion solution consisting of 83.33% by volume of University of Wisconsin (UW) solution (K_2HPO_4, 3.38 mg/ml; KH_2PO_4, 8.16 mg/ml; KCl, 1.23 mg/ml; $NaHCO_3$, 0.45 mg/ml), 8.33% by volume of albumin (Albutein; Alpha Therapeutic Corporation, Los Angeles, CA) and 8.33% by volume of 50%-dextrose injection, (Abbott Laboratories, North Chicago, IL). The vascular pedicle was then clamped at the level of the hilum for a total ischemic time of 45 min. During that time, the involved vessels were repaired with 7-0 monofilament suture (Proline; Ethicon Inc., Somerville, NJ) and the other clamps released.

In the treatment group, 1 ml of sPSGL (5 μg/ml) in 1 ml UW solution was perfused slowly (2–3 min) into the left kidney after perfusion with 9 ml of perfusion solution, and the vascular pedicle was clamped for 45 min; this allowed sPSGL to block P-selectin during the actual period of ischemia. Additional material (50 μg) was then injected intravenously into the animal after 3 h of reperfusion to block E-selectin before its peak at 6 h (data not shown). This protocol was determined by numerous preliminary experiments with various sPSGL doses to assess the most effective blockade of PMN infiltration and E-selectin expression (data not shown). Blood samples (0.5 ml) for creatinine determination were obtained from the tail vein at 0, 1, 2, and 3 d after reperfusion. Creatinine was measured by a modified Jaffe's reaction on an autoanalyzer (911; Hitachi, Indianapolis, IN).

sPSGL. sPSGL.I316 (Genetics Institute, Cambridge, MA) is a recombinant soluble form of P-selectin glycoprotein ligand-1 (PSGL-1) (9). The mammalian expression vector pED (10) was liganded to a cDNA encoding sPSGL.I316 which comprises the mature extracellular domain of PSGL-1, truncated at the isoleucine molecule at position 316. This vector was stably transfected and amplified in a DHFR-Chinese hamster ovary cell line stably transfected with vector pMT4neo expressing both the cDNA encoding an α(1, 3/1, 4) fucosyltransferase (11) and a cDNA encoding core 2 β-1,6-*N*-acetylyglucosaminyltransferase (12). The secreted sPSGL.I316 was purified from serum-free Chinese hamster ovary cell conditioned medium by application to a toyopearl column (QAE 500C; TosoHAAS, Montgomeryville, PA). The column was washed using 5 column vol of 25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer. After sixfold concentration by ultrafiltration/diafiltration using tangential flow membrane unit (Millipore Corp., Bedford, MA) and 1 M NaCl/25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer, the concentrated material was applied to a hydroxyapatite column (Pharmacia Biotech, Piscataway, NJ) equilibrated with 1 M NaCl/25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer, washed with 15 column vol of 150 mM NaCl/25 mM Tris/1 mM $CaCl_2$, pH 7.4, buffer and eluted using 30 mM $NaPO_4$/150 mM NaCl, pH 7.4. The eluate was run through a Sephacryl S-300 HR sizing column equilibrated in PBS, pH 7.2. Collected fractions were analyzed by SDS-PAGE using Alcian blue/silver staining (Fig. 1). sPSGL-containing fractions were assayed for selectin binding activity as described (13). Endotoxin levels were determined to < 2 endotoxin U/mg.

Protease-treated sPSGL. sPSGL.I316 (2.5 mg/ml) was incubated with or without 10 μg/ml mocarhagin (Sigma Chemical Co., St. Louis, MO) in PBS/1 mM $CaCl_2$ at 37°C for 6 h (14). Mocarhagin, a protease isolated from cobra venom, was inactivated by adding EDTA to 2 mM and heating to 85°C for 5 min. Buffer with only mocarhagin was also included as an additional control. Cleavage of amino-terminal 10 amino acids of mature sPSGL was confirmed by Western blot using monoclonal PSL275 (Genetics Institute) which binds to the amino-terminal portion of mature PSGL-1 (15), or monoclonal PSL1H3 (Genetics Institute), which binds to an epitope proximal to the carboxyl terminus of sPSGL.I316 (Fig. 2). Inactivation of P-selectin binding was confirmed by binding to soluble P-selectin in competitive ELISA format.

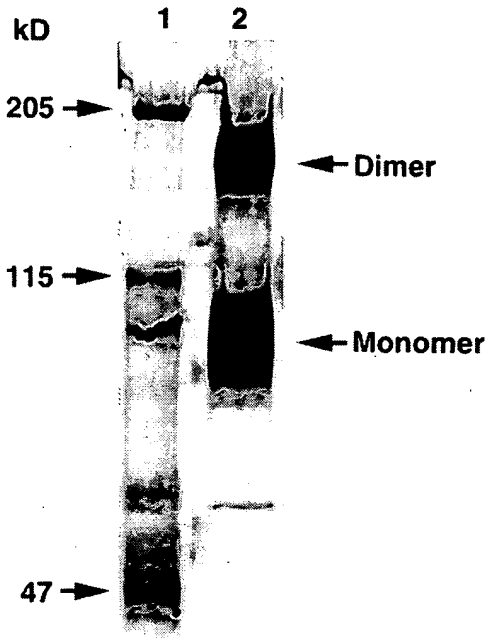

Figure 1. Nonreducing 4–20% polyacrylamide-SDS gel of 1 μg purified recombinant sPSGL.I316. The gel is developed with Alcian blue and silver stain. The purified sPSGL glycoprotein appears as both a dimer and monomer exhibiting an average approximate relative molecular mass of 190 and 95 kD, respectively. Lane *1*, size marker; lane *2*, sPSGL.

Immunohistology. Representative portions of the kidneys were snap-frozen in liquid nitrogen at 0, 3, 6, and 12 h and at 1, 3, 5, and 7 d after operation. The contralateral kidneys removed from the animal at the time of ischemic injury were used as controls. Sections (4 μm) were fixed in acetone for 10 min, air-dried, and stained individually with mouse anti–rat monoclonal or polyclonal antibodies. PMNs were stained with a rabbit anti–rat PMN FITC-conjugated gamma globulin (Inter Cell Technologies, Inc., Hopewell, NJ). Stained cells were counted under a fluorescence microscope (UFX-IIA; Nikon, Garden City, NY) and expressed as cells per glomerulus (×400, > 40 fields counted/specimen, 2–3 specimens/kidney). The specimens were also stained with ED-1 for macrophages, W3/25 for $CD4^+$ cells, OX-8 for $CD8^+$ cells, and OX-3 for MHC class II (Bioproducts for Science, Inc., Indianapolis, IN). The sections were then interacted with rabbit anti–mouse IgG by the alkaline phosphatase, anti–alkaline phosphatase method and counterstained with hematoxylin (16). Stained cells were then counted on an ocular grid and marker positive cells expressed as mean±standard deviation (M±SD) of cells per field of view (cells/FV, ×400, > 30 fields counted/specimen, 2–3 specimens/kidney). For morphology, formalin-fixed sections were stained with periodic-acid-Schiff.

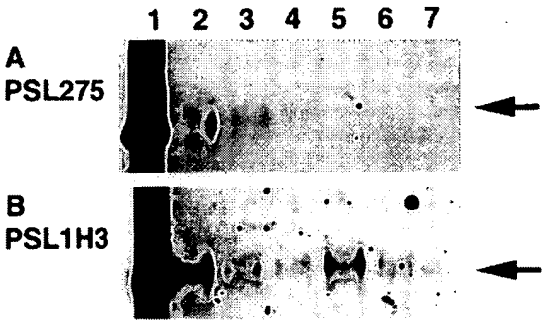

Figure 2. Western blot of various amounts of recombinant sPSGL. (*A*) Undigested (lanes *2–4*) or digested with mocarhagin (lanes *5–7*) using amino-terminal specific mAb PSL275. Amounts of sPSGL loaded onto each lane are 100 ng (lanes *2* and *5*), 50 ng (lanes *3* and *6*), 25 ng (lanes *4* and *7*), respectively. Lane *1*, size marker. (*B*) Same as *A* using a proximal to carboxyl-terminal specific mAb PSL1H3. Note that protease-digested sPSGL (lanes *5–7*) has lost its amino terminus for P-selectin binding (*A*).

Reverse transcriptase polymerase chain reaction (RT-PCR). The expression of various adhesion molecules, cytokines, and growth factors was examined by RT-PCR (17–19) at 0, 3, 6, 12, 24, and 48 h and at 3, 5, and 7 d. Total RNA of kidney specimens was extracted by the guanidine isothiocyanate method (Ultraspec RNA kit; Biotecx Laboratories, Inc., Houston, TX) (20). The total RNA was air-dried, resuspended in diethyl pyrocarbonate–treated water, and the approximate quantity of RNA was determined with an OD 260 reading. The purity of the RNA was assessed with an OD 260/280 ratio, confirming all samples > 1.8. Total RNA (2.5 μg) was used for first-strand cDNA synthesis employing 1.2 μg of oligo dT_{12-18} and the Superscript reverse transcriptase method according to supplier-recommended conditions (GIBCO-BRL, Gaithersburg, MD). Nonlooping nonoverlapping oligonucleotide primer pairs from separate exons were prepared for each gene studied by Clontech (Palo Alto, CA) or Genosys Biotechnologies (The Woodlands, TX). The specific primers (IL-2, IL-2R, IL-4, IFNγ, IL-1, IL-6, TGFβ, TNFα, RANTES, MCP-1, and β-actin) were used as described (21). The sequences for the E-selectin, C3, and C1 specific primer are as follows: E-selectin: upstream primer, 5′-TCTTTAAGCTCAAGGAAT-3′; downstream primer, 5′-TACCGATCGTGCAGGTCA-3′; C3: upstream primer, 5′-CGGAATCGATTGCCTATT-3′; downstream primer, 5′-CCGTCTTGGTCCAACTTA-3′; C1: upstream primer, 5′-GTCCGATTCGCGTTGCA-3′; downstream primer, 5′-TAACGCATTTCATCGGAC-3′.

The competitive PCR for quantification of mRNA was performed as described (19). For each 25-μl amplification, 2.5 μl of first-strand cDNA product was used. 2–16-fold serial dilutions of known quantities of PCR MIMICS were added to the PCR reaction containing constant amounts of sample target cDNA. PCR products (5 μl) were run on 1.5% agarose gel and stained with ethidium bromide, then gene-specific bands were visualized with ultraviolet light (Fig. 3). The quantities of MIMICS and target cDNA were compared using a PC SCANJET with analysis by Adobe Photoshop software (Adobe Inc., Mountain View, CA). Tissues were tested at all time points for an individual cytokine mRNA transcript as well as for the control β-actin mRNA transcript. Scanner analysis of photographs of the DNA-stained agarose gels was evaluated by the band intensity comparison of β-actin expression versus each cytokine expression in computer image analysis. Each PCR reaction was repeated twice in all three kidney specimens in each time group and were not appreciably

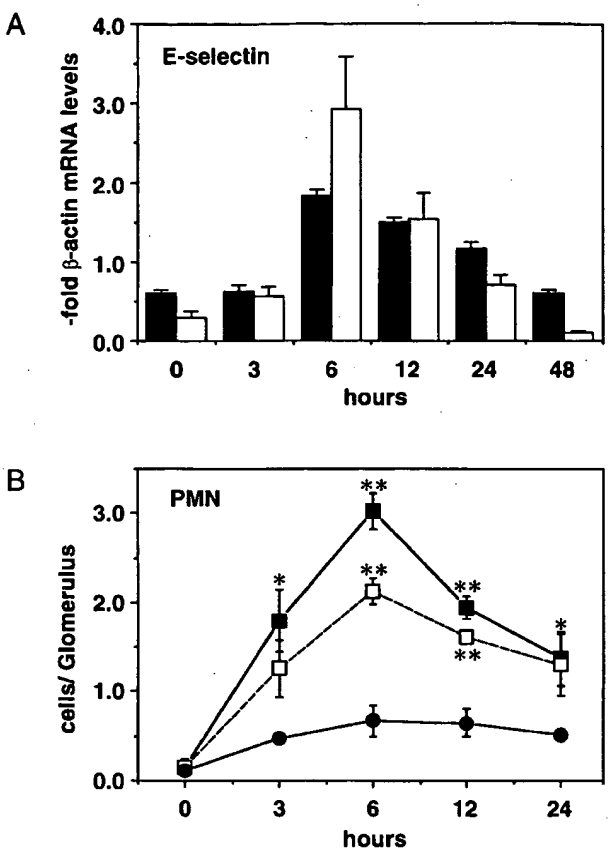

Figure 4. Patterns of E-selectin mRNA expression and PMN infiltration in rat ischemic kidneys are shown. (*A*) E-selectin mRNA expression is upregulated both in warm (■) and cold (□) ischemia. (*B*) PMNs infiltrate the organs in parallel. $*P < 0.05$; $**P < 0.01$ compared with control kidneys (●). Data are expressed as mean±SEM.

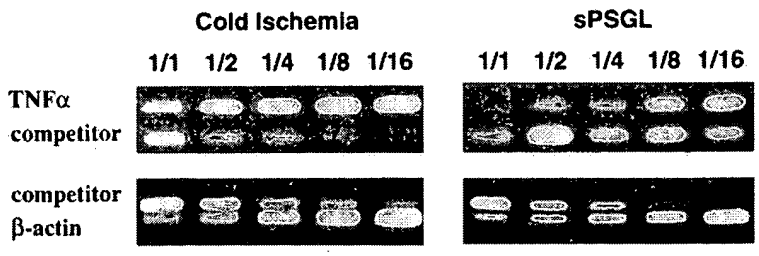

Figure 3. Examples of semiquantification by competitive RT-PCR assay. TNFα mRNA expression in a cold ischemic kidney and in a kidney at 3 d after sPSGL treatment is compared. Expression of TNFα and β-actin control mRNA were quantitated by the amount of competitive template that most nearly equaled the amplification of cellular cDNA. Compared to β-actin expression (equivalent point is 1/2), TNFα expression of cold ischemia is 2.0 (equivalent point is 1/1), and that of sPSGL-treated animal is 0.25 (equivalent point is 1/8) in this sample.

different from one another. This method has been noted as accurate as scintillation counting of ^{32}P-labeled PCR products (22).

Statistical analysis. The results are expressed as arithmetic means (±SEM). Statistical comparisons between groups were performed by Student's *t* test. The difference was considered to be significant when $P < 0.05$.

Results

Cellular, molecular, and morphological changes after I/R injury. Inflammatory changes became apparent in the single injured kidneys in uninephrectomized animals within a few hours after I/R. The expression of E-selectin mRNA peaked at 6 h after both warm ischemia and cold ischemia and was still upregulated at 12 h before declining to baseline (Fig. 4 *A*). Rapid migration of PMNs into the organ occurred in parallel, more intense in warm than in cold injury, with the cells localizing preferentially in and around glomeruli as well as throughout the interstitium. Few PMNs were ever noted in the uninjured kidneys of uninephrectomized controls (Fig. 4 *B*).

Within 24 h of the warm I/R insult, intercellular adhesion molecule (ICAM)-1 mRNA expression peaked and remained upregulated for 48 h before declining. Cold perfusion substantially inhibited expression of this adhesion molecule (Fig. 5 *A*). The transcripts of the complement component, C3, were highly upregulated by 12 h after warm ischemia, somewhat less in the cold ischemia kidneys. C1 remained minimally expressed (Fig. 5 *B*).

Lymphocytes and macrophages, as determined by immunohistology, began to infiltrate the affected organs coincident with ICAM-1 expression at 1–3 d. Cold perfusion decreased but did not abolish the cellular infiltrate (Fig. 6). ED-1$^+$ macrophages entered the kidneys after 24 h and remained obvious throughout the 7-d follow-up period. Relatively large numbers of CD4$^+$ leukocytes infiltrated the organs in a similar pattern, peaking at 5 d; CD8$^+$ lymphocytes were a minor feature of this injury. Importantly, MHC class II expression, noted in the interstitium, collecting tubules, and on periglomerular cells, was upregulated in parallel with the presence of CD4$^+$ cells.

Cytokine mRNA production increased substantially after the I/R insult (Fig. 7). Most T cell–derived cytokines (IL-2 and IL-4) peaked between 3 and 5 d, at which time numbers of infiltrating CD4$^+$ lymphocytes were obvious; IL-2R and TNFα, expressed by activated T cells, increased in parallel. RANTES, a macrophage chemoattractant derived from T cells and other cell populations, remained upregulated in a more sustained fashion throughout the follow-up period; its presence correlated with the broad peak of infiltrating macrophages (Fig. 7 *A*). IFNγ increased slowly, but seemed less of a feature in the process than the other T cell factors. The macrophage associated products, IL-6 and TGFβ, had become highly expressed within 3 d; IL-1 increased gradually (Fig. 7 *B*). Little expres-

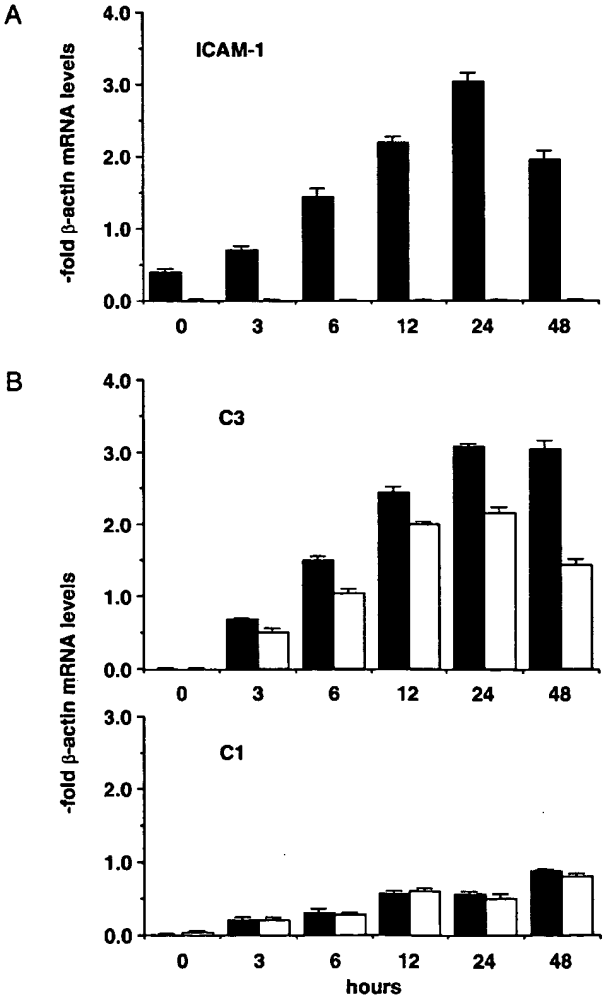

Figure 5. ICAM-1 and complement mRNA expression after warm and cold ischemia. (*A*) High mRNA expression of ICAM-1 in warm ischemia (■) and negligible expression in cold perfused organs (□) is noted. (*B*) C3 is highly expressed after both warm and cold ischemia; C1 is minimally expressed.

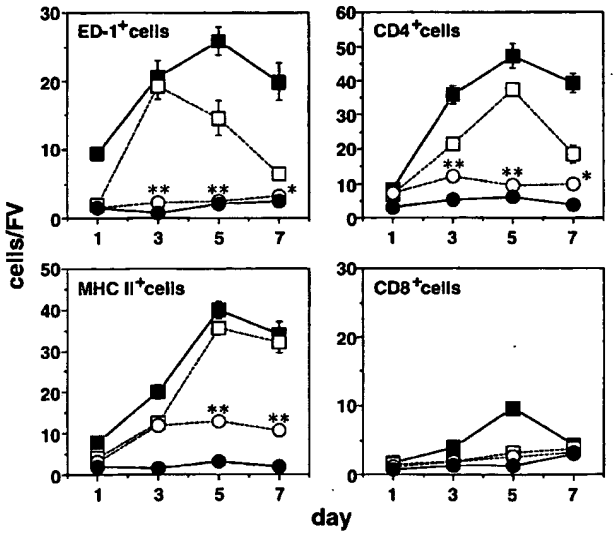

Figure 6. Cell infiltration into kidneys after warm (■) and cold (□) ischemic injury is increased. Treatment with sPSGL (○) inhibits cell migration, similar to that in uninephrectomized controls (●). $^*P < 0.05$; $^{**}P < 0.01$ compared with sPSGL treatment and cold ischemia.

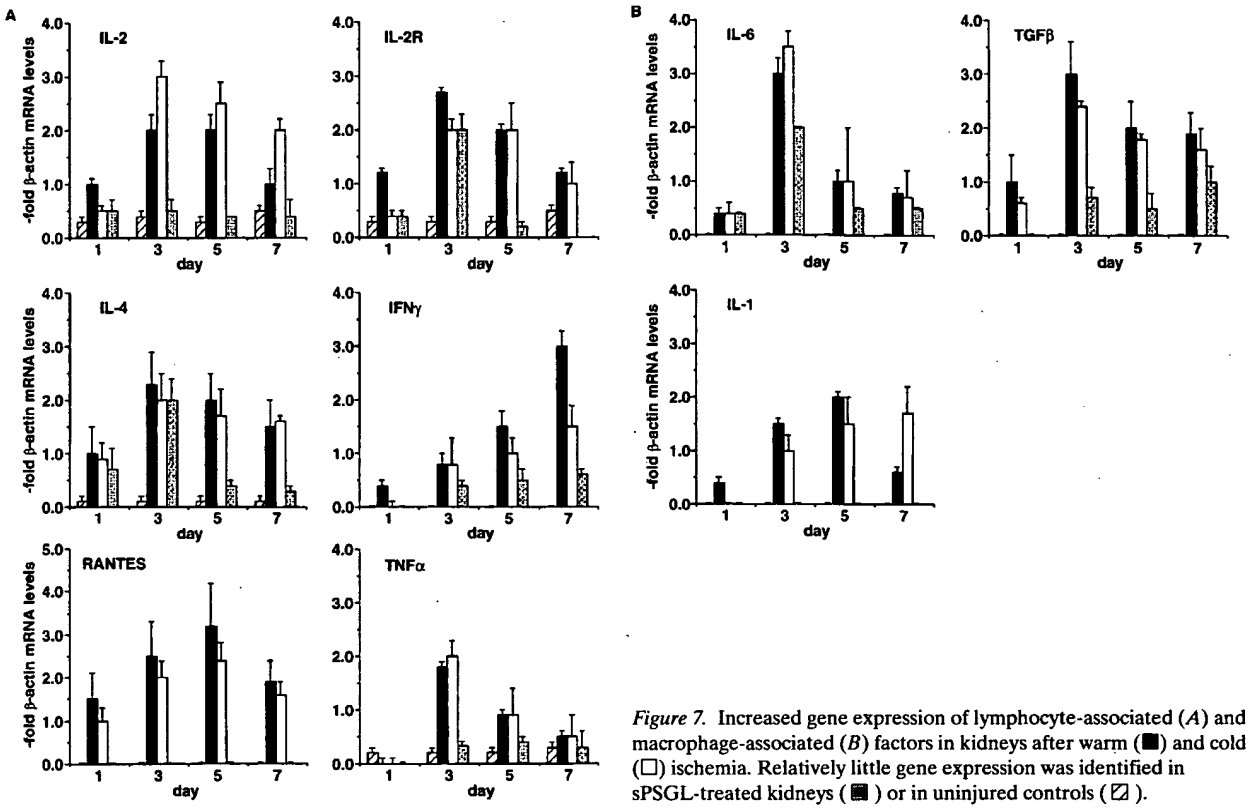

Figure 7. Increased gene expression of lymphocyte-associated (*A*) and macrophage-associated (*B*) factors in kidneys after warm (■) and cold (□) ischemia. Relatively little gene expression was identified in sPSGL-treated kidneys (▩) or in uninjured controls (▨).

sion of cells or cell products was identified in uninjured uninephrectomized control kidneys.

The kidneys injured by ischemia were then assessed at day 3. Tubular necrosis was the most obvious effect of warm and cold ischemic injury (Fig. 8, *B* and *D*, respectively), with interstitial edema, denudation of tubular cells, and tubular casts compared to uninephrectomized controls (Fig. 8 *A*). Such changes were more severe after warm ischemia, but also surprisingly obvious after cold ischemia.

Effects of sPSGL on I/R injury. Cold ischemic organs were perfused with sPSGL to block P-selectin, and the animal was then given the material intravenously to inhibit E-selectin before its peak expression at 6 h. E-selectin expression in sPSGL-treated rats remained at baseline, comparable to that of β-actin controls (Fig. 9 *A*). At the same time, PMN infiltration into both glomeruli and interstitium was reduced by ~ 50% (Fig. 9 *B*). Neither protease-treated sPSGL with cleavage of P-selectin binding by mocarhagin nor buffer perfusion at 0 h and injection at 3 h affected E-selectin expression and PMN infiltration, emphasizing that the structural features of sPSGL are essential to its action.

Morphologically, sPSGL treatment improved renal damage substantially compared to the cold ischemia alone, with minimal evidence of tubular compromise or interstitial edema (Fig. 8 *C*). Immunohistologically, sPSGL reduced numbers of infiltrating ED-1$^+$ macrophages, CD4$^+$ T cells and MHC class II$^+$ cells toward baseline; indeed, these cells were virtually absent in the injured but treated organs and at the same level as kidneys in uninephrectomized controls (Fig. 6). Similarly, the mRNA expression of inflammatory cytokines was diminished, with IL-2, IFNγ, and RANTES completely inhibited by sPSGL treatment. IL-2R and IL-4 were less affected (Fig. 7 *A*). Expression of the macrophage-associated factors, IL-1, TNFα, TGFβ, and NOSi, was diminished, although IL-6 still showed some upregulation at 3 d (Fig. 7 *B*).

Effect of sPSGL on renal function after I/R injury. To ascertain whether the decrease in infiltrating cells and their products in the injured kidneys affected function, levels of plasma creatinine were monitored for 3 d after warm and cold I/R injury, and after treatment with sPSGL. Rats with ischemic kidneys showed significant elevation of plasma creatinine levels by 2 d compared with uninjured controls. Treatment with sPSGL protected against this transient dysfunction; no increase in creatinine was noted (Fig. 10).

Discussion

Increasing clinical evidence both from single centers and from United Network of Organ Sharing has supported a relationship between delayed initial graft function and acute rejection (1–3). The significance of this antigen-independent injury on later graft survival remains conjectural, with most series suggesting that its primary effects are limited to the first year after transplantation. However, it seems increasingly clear that initial dysfunction plus early acute rejection in combination interact to reduce long-term graft success substantially (4). This seemingly synergistic effect may be explained in practical terms by the difficulties in recognizing and treating acute rejections in patients with nonfunctioning grafts. It has also been noted in experimental models, including data from this study,

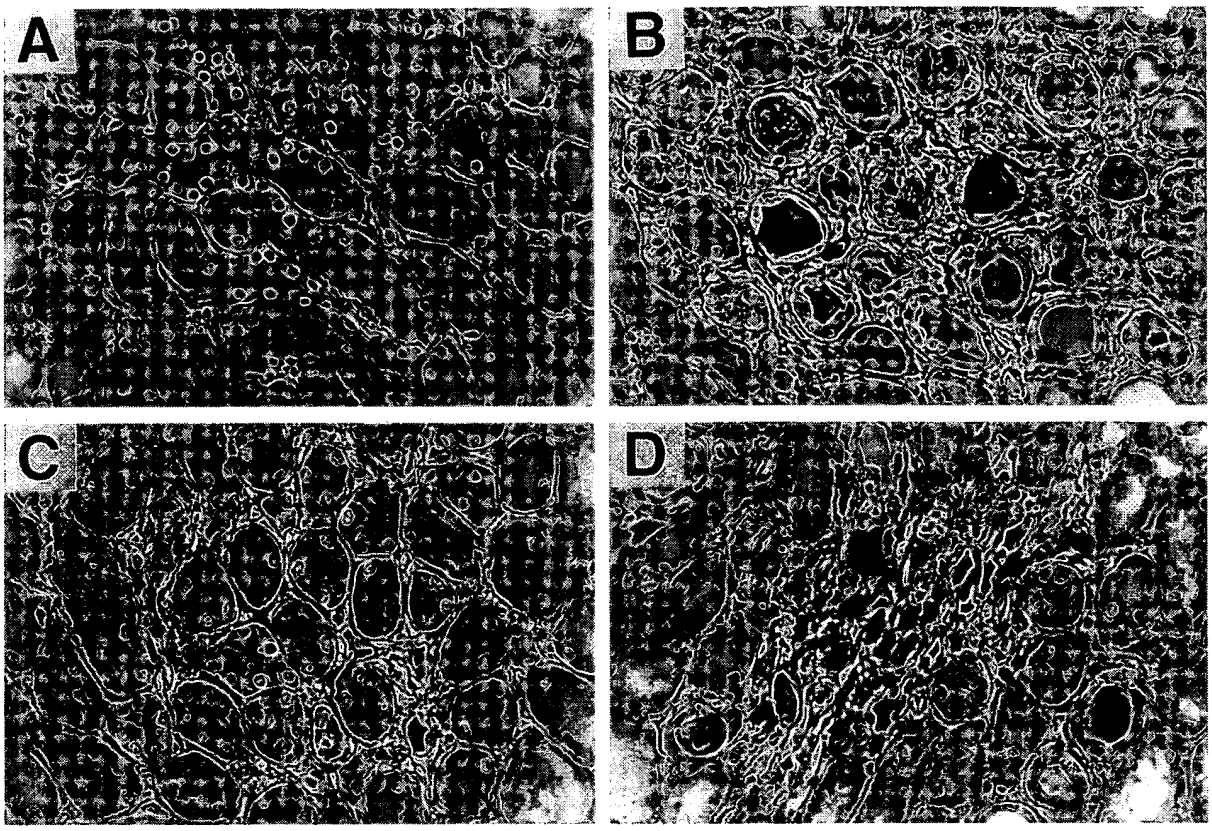

Figure 8. Histological features of ischemic kidneys (day 3). Significant tubular necrosis developed after both warm (*B*) and cold (*D*) ischemia. After sPSGL treatment (*C*) tubules are minimally damaged. (*A*) Control uninephrectomized kidney. Periodic-acid-Schiff staining, ×400.

that an ischemic insult may increase the immunogenicity of the organ by upregulating HLA class II antigens.

Because detailed knowledge about the cellular and molecular dynamics occurring in the kidney after I/R injury is relatively limited (5), we have examined the early events occurring in a well defined model. It appears that adhesion molecules, infiltrations of cell populations, and their associated products are highly expressed after warm ischemia and are still obvious, albeit less intensely, after cold perfusion. This finding suggests that despite cold preservation of the organ before actual engraftment, the transplanted kidney may not be as protected as thought hitherto, and may harbor potential triggering mechanisms for later immunologic host events.

P-selectin is the key adhesion molecule involved in the earliest events in the adherence of circulating leukocytes in rolling to tissues in inflammatory states (23, 24). It is constitutively present in the membranes of alpha granules of platelets and the Weibel-Palade bodies of endothelial cells, and is translocated to the plasma membrane of these cells in response to various stimuli (25–28). It mediates the adhesion of PMNs or monocytes to activated platelets or endothelial cells (29, 30), and the rolling of leukocytes to activated endothelial cells (31). P-selectin also plays a role in inflammatory and thrombotic disorders, including I/R injury (32, 33), leukocyte adhesion to lung endothelial cells in rats after cobra venom factor infusion (34), and leukocyte accumulation in thrombogenic grafts (35). The molecule has an important role not only in earliest cellular responses but in chronic inflammation as well. Monocyte accumulation is diminished substantially at later time points after drug-induced peritonitis (36), and in T cell–dependent contact hypersensitivity responses in P-selectin knockout mice (37). P-selectin also mediates monocyte–microvascular interaction in rheumatoid synovitis (38).

Among several ligands for the selectin families (39), P-selectin's high-affinity counter receptor, PSGL-1, is probably the most extensively characterized (40). The effects of blocking antibodies to P-selectin also indicate that the binding of this molecule to its ligand is important in modulating I/R injury (32, 33).

Translocation of stored P-selectin to the plasma membranes of endothelial cells occurs early after activation. In contrast to P-selectin, which is already produced and stored within the endothelial cells, E-selectin is expressed de novo on endothelial cells only after the transcriptional induction of E-selectin mRNA by proinflammatory agents such as TNFα or IL-1β in vitro (7, 8, 41). Upregulation of E-selectin on endothelial cells of renal allografts has been seen in biopsy specimens which show acute rejection (42). In this study, we have used this induction of E-selectin transcription and the PMN infiltration into glomeruli as measures of the local inflammatory status of renal endothelial cells. The peak mRNA expressions of E-selectin and PMN infiltration at 6 h after reperfusion were used to determine the dosage protocol.

Several in vitro studies have demonstrated that PSGL-1

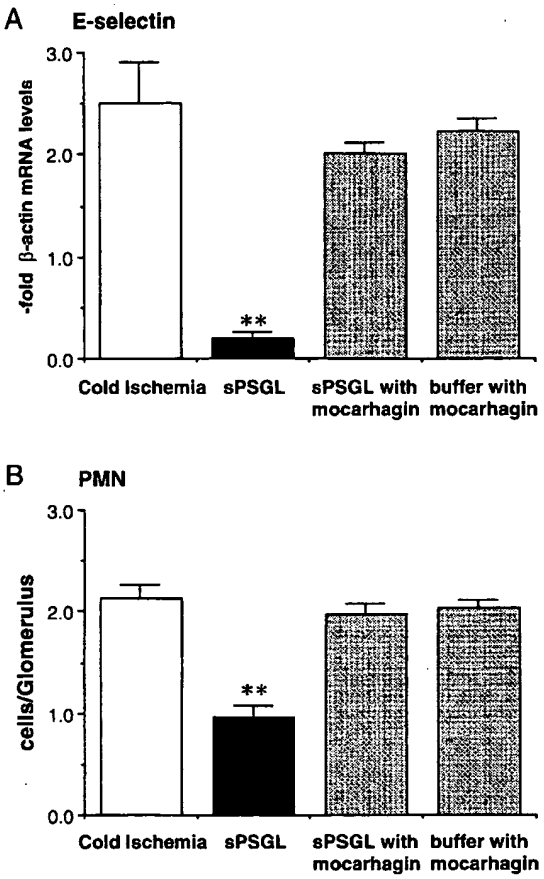

Figure 9. Inhibitory effects of sPSGL treatment on E-selectin mRNA expression and PMN infiltration. (*A*) E-selectin expression in cold perfused kidneys was inhibited by sPSGL without mocarhagin (■). No inhibition was observed after treatment with sPSGL with mocarhagin and buffer with mocarhagin (■). (*B*) PMN infiltration in cold ischemia was also diminished with sPSGL without mocarhagin (■). No decrease in cell numbers was observed by sPSGL with mocarhagin and buffer with mocarhagin (■). $^{**}P < 0.01$. Data are expressed as mean±SEM.

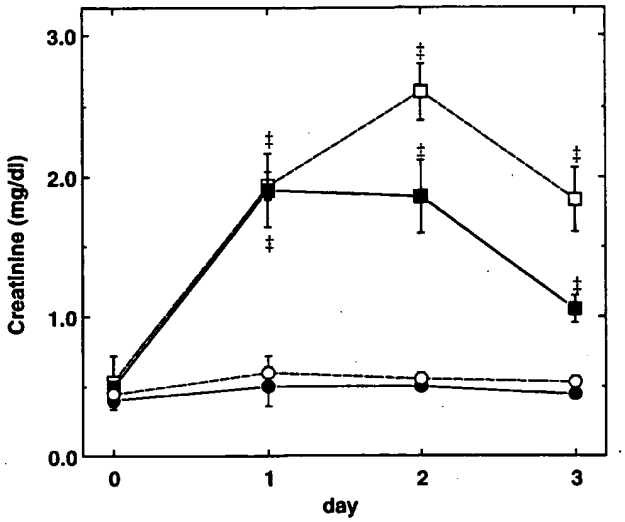

Figure 10. Increased plasma creatinine levels after warm (■) and cold (□) ischemia. Data are expressed as mean±SEM for each group ($n = 4$). $^{\ddagger}P < 0.005$ compared with sPSGL treatment (○) and normal kidneys (●).

can serve as a ligand for all three selectin family members: P-, E-, and L-selectin (9, 43–46). A recombinant soluble form of PSGL-1, sPSGL, was used in these experiments to investigate the role of these molecules. Preliminary pharmacokinetic analysis estimated the postdistributional half-life of sPSGL in rats to be ~ 2 h (data not shown). The dosage protocol was designed to provide an adequate concentration of the blocking ligand during the ischemic period. Accordingly, a low dose of sPSGL was added to the final perfusion of cold UW solution to bind any P-selectin upregulated on renal endothelium during the 45-min ischemic period. Recent studies have shown that P-selectin expression on endothelial cells can also be regulated at the transcriptional level, with agents such as LPS, TNFα, or IL-3 inducing a later and more chronic expression of P-selectin (28, 47, 48). With this in mind, a second systemic dose was given 3 h after reperfusion to bind selectin molecules expressed at this later time point.

Because it is a specific ligand for selectins, sPSGL presumably acts by inhibiting the binding between endothelial cells (E-/P-selectin) and PMNs, and/or L-selectin–mediated PMN aggregation (9, 13, 15, 46, 48). In this study, sPSGL was found not only to block PMN infiltration but also to partially block the induction of E-selectin mRNA in the kidney. To confirm that this effect was associated with the blocking of P-selectin and not E-selectin, sPSGL was digested with the snake venom protease, mocarhagin. Mocarhagin-treated sPSGL loses the capacity to bind P-selectin, yet maintains the capacity to bind E-selectin (14). Animals treated with this protease-treated sPSGL failed to block both E-selectin induction and PMN influx 6 h after reperfusion (Fig. 9). This result strongly suggests that the mode of action for sPSGL on these early phase events is predominantly mediated by blocking P-selectin. One possible model of this finding is that by inhibiting the initial P-selectin–mediated tethering of leukocytes to endothelium, sPSGL diminishes the localized production of proinflammatory cytokines which induce E-selectin expression. This theory is supported by the low influx of PMN into tissues of P-selectin–deficient mice (49). Transplantation of hearts from such donors to wild-type recipients demonstrates a reduction of PMN infiltration and increase of graft survival (50), emphasizing the critical role of this selectin in the initial cell extravasation and infiltration. Moreover, PMN rolling is inhibited by anti–PSGL-1 antibody in vivo (51). It is unclear from the present data whether the later phase blocking effects which sPSGL treatment causes on the cytokine-adhesion cascade can be attributed to possible additional E- or L-selectin blockade by sPSGL. This will require further study. However, it is noteworthy that efforts by other investigators to ascribe a significant role for L-selectin in renal I/R injury, using L-selectin–deficient mice, have thus far failed (52).

De novo mRNA expression of ICAM-1 follows that of the selectins in the ischemic organs, peaking at 24 h in the warm ischemia model. I/R injury is reported to increase ICAM-1 expression (53). Cold I/R injury showed no de novo ICAM-1 expression compared to warm I/R injury, indicating the greater

influence of the warm injury. However, despite no ICAM-1 expression, sequential events still occurred, suggesting the effectiveness of alternative adhesion molecules in cold I/R. It has been reported that anti–ICAM-1 antibody can inhibit the transient renal dysfunction which occurs after warm ischemia (54). As noted in the present studies, sPSGL treatment also protects against this transient renal failure by interfering at an earlier phase of cell adhesion than anti–ICAM-1 antibody.

Activation of complement after I/R injury presumably increases the permeability of vascular walls (55). Activation of the complement cascade, whether the classical or alternative pathway, finally leads to the cleaving of C3, which plays a role in the adhesion of PMNs and monocyte/macrophages to endothelium (56). The presence of C3 mRNA has been identified in rejecting human kidney grafts (57). The rapidly increased expression of C3 in these experiments may contribute to cell infiltration into the injured kidney. The negligible mRNA expression of C1 after both warm and cold ischemia suggests this component is less important in this context.

Both MHC class II expression and subsequent cell migration to the injured kidney were suppressed by sPSGL. This putatively diminished immunogenicity of the organ may have important clinical ramifications, as it may decrease the incidence of acute rejection in grafts with delayed function (2). It has been reported that the kinetics of IFNγ induction of MHC class II genes occurs slowly, with mRNA levels peaking at 24–48 h (58). The pattern of IFNγ expression is well explained by the sequential expression of MHC class II in the present I/R model. Moreover, IFNγ can be potentiated by TNFα produced by PMNs and other cell populations (59). This IFNγ mRNA expression was also diminished by sPSGL. Other lymphocyte-derived cytokines inhibited by sPSGL include IL-2, TNFα, and RANTES (Fig. 7 *A*). RANTES may facilitate T cell and macrophage accumulation by direct chemotactic effects or by regulating the expression of macrophage products. The relatively prolonged expression of this factor after I/R injury has also been noted in a model of chronic allograft rejection (21). This and macrophage-associated cytokines (IL-1, IL-6, and TGFβ) were reduced after treatment.

These data all imply that minimal cytokine activity occurs once initial PMN adhesion, via P-selectin to graft endothelium which presumably triggers an inflammatory event, is blocked. Damage to the glomerulus and tubular cells caused by I/R is inhibited by blocking initial PMN adhesion. As a result, the transient renal dysfunction that follows ischemia can be prevented. These findings emphasize the role of various host cell populations and their products in the injury of I/R. Their early inhibition may potentially influence both short- and long-term survival of renal allografts.

Acknowledgments

This work was supported by U.S. Public Health Service grant 9 R01 DK 46190-23.

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